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Methylene Blue Inhibits the Antithrombotic Effect of Nitroglycerin

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Objectives. The aim of this study was to examine whether cyclic guanosine monophosphate (GMP) may be involved in the antithrombotic action of nitroglycerin.

Background. Nitroglycerin has been shown to inhibit platelet function in vitro by stimulating prostacyclin or inhibiting thromboxane A_2 production, or both. Nitroglycerin has also been shown to possess potent antithrombotic properties in vivo. However, the mechanism of this antithrombotic effect is unclear.

Methods. Nitroglycerin was infused to produce a 10% decrease in mean arterial pressure in 27 normal pigs by exposing their circulating arterial blood to porcine aortic media in an ex vivo perfusion chamber. Eight pigs received an infusion of nitroglycerin alone; eight received an infusion of methylene blue, a guanylate cyclase inhibitor, followed by nitroglycerin infusion and five pigs received an infusion of nitroglycerin followed by methylene blue and subsequent infusion of cyclic GMP.

Results. With nitroglycerin alone, quantitative autologous indium-111–labeled platelet deposition ($\times 10^6$) on the aortic media was decreased to 63.9 ± 10.4% ($p = 0.01$) of the baseline control platelet deposition. Methylene blue given before nitroglycerin tended to increase platelet deposition relative to baseline and platelet deposition after nitroglycerin was 142 ± 35% (p = NS) of baseline value. In pigs that received all three agents, nitroglycerin reduced platelet deposition to 42.3 ± 12.2% of baseline value; this decrease was then attenuated by subsequent methylene blue infusion but was enhanced by cyclic GMP infusion to 16.4 ± 3.8% of baseline value ($p = 0.006$ vs. baseline control and $p = 0.02$ versus methylene blue infusion).

Conclusions. Guanylate cyclase inhibition with methylene blue abolishes the antithrombotic effect of nitroglycerin, which can be enhanced by cyclic GMP.

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Organic nitrates have been used to prevent and treat myocardial ischemia for more than a century. Nitroglycerin's proved clinical efficacy is believed to be due to vasodilation of both the coronary and the peripheral vascular beds (1–5). In addition, nitroglycerin has been shown to inhibit platelet aggregation in vitro (6–8), although this effect is observed at concentrations that are not achievable in vivo in humans (7). Recently, however, we demonstrated (9) that nitroglycerin infused to decrease mean arterial pressure by 10%, as it is given in patients, causes a significant decrease in platelet mural thrombus deposition and the associated vasoconstriction related to arterial injury in the intact animal. These data suggested that nitroglycerin may inhibit the platelet–arterial wall interaction in vivo and may decrease injury-related vasoconstriction by inhibiting platelet-dependent vasoconstriction as well as by a direct effect on the arterial smooth muscle (9,10). Subsequently, we demonstrated (11) in humans that nitroglycerin infused to lower mean arterial pressure by 10% is associated with significant platelet inhibition.

The mechanism by which nitroglycerin inhibits this platelet–arterial wall interaction in vivo is not clear. In vitro, at supraphysiologic concentrations, it appears that the inhibition of platelet interaction by nitroglycerin is mediated by stimulation of prostacyclin or inhibition of thromboxane A_2, or both (6–8). However, at nitroglycerin concentrations that are clinically relevant, the beneficial effects of nitrates do not appear to be mediated by an increased prostacyclin production (12,13). More recently, in a dog model of stenosed canine coronary arteries, it was shown (14) that cyclic blood flow responses due to platelet aggregation and dislodgment can be inhibited by clinically achievable nitroglycerin concentration in vivo, and that this may be mediated by cyclic guanosine monophosphate (GMP).

To investigate whether cyclic GMP may be involved in the inhibition by nitroglycerin of platelet-mural thrombus deposition on deeply injured vessel wall exposing the media (9), we examined the effects of nitroglycerin on quantitative platelet deposition onto aortic media in the presence or absence of methylene blue, a guanylate cyclase inhibitor, and the administration of cyclic GMP.

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Methods

Experimental procedure. Normal Yorkshire pigs approximately 6 weeks of age were anesthetized by an intramuscular injection of a mixture of 100 mg of ketamine (Ketalar, Parke-Davis Canada Inc.) and 80 mg of azaperone (Stresnil, Janssen Pharmaceutical). A cutdown of the femoral artery and vein was then performed with the artery and vein then cannulated and connected to superfusion flow chambers with silicone tubing, 6 to 10 in. (7.6 to 12 cm) long. Blood was drawn from the femoral artery into the chambers to interact with aortic media strips, by means of a peristaltic pump (Masterflex, model 7013) placed distal to the superfusion flow chambers (15,16). A 5-min superfusion was performed at a constant blood flow of 20 ml/min through the chamber, after which the aortic media was removed and the amount of radioactively labeled platelets interacting with the media was measured in a gamma counter. Duplicate runs through the flow chambers were performed for each baseline control and drug infusion and the mean counts noted. The arterial blood pressure was monitored during the experiment. The experiments were performed in accordance with the "Position of the American Heart Association on Research Animal Use."

Radioactive labeling of platelets. Approximately 16 to 24 h before the superfusion experiments, 43 ml of blood in 7 ml of acid-citrate dextrose solution was obtained from the pig by venipuncture. The blood was centrifuged at 180 *g* for 15 min, after which the platelet-rich plasma was removed and centrifuged at 1,600 *g* for 10 min. The platelet-poor plasma was set aside while the platelet-rich pellet was resuspended with indium-111 (^{111}In)-tropolone and 2 ml of acid-citrate dextrose saline solution at room temperature for 20 minutes. The ^{111}In-tropolone mixture was prepared by mixing 50 μg of tropolone (Sigma Chemicals) in normal saline solution with 0.5 mCi of ^{111}In chloride (Amersham). After the 20-min incubation, the suspension was centrifuged for 10 min at 1,600 *g*. The platelet-containing pellet was resuspended in 4 ml of platelet-poor plasma and incubated for another 5 min to remove any free unbound ^{111}In-tropolone. It was again centrifuged for 10 min at 1,600 *g* and the labeled platelet pellet was finally resuspended in 5.5 ml of platelet-poor plasma and centrifuged at 100 *g* for 10 min to remove any macroaggregates. The ^{111}In-labeled platelets now contained in the supernatant were removed, and counted in an ionization chamber before intravenous reinjection into the pig. The superfusion experiment was performed 16 to 24 h later, to allow a return to baseline steady state conditions after the labeled platelet infusion.

Superfusion chamber. The superfusion chamber developed by L. Badimon (15,16) was designed to mimic the tubelike shape of the vascular system. It was made of Plexiglas and consisted of an upper lid and a lower block, the lower block having a small cylindric hole of 1-mm diameter bored through it to allow the flow of blood. The surrounding upper wall or roof of this cylindric tube (1-mm diameter × 30 mm long) was removed, resulting in a window permitting direct exposure of flowing blood to a piece of exposed arterial media held in place by the pressure of the upper lid and secured together by a surrounding chamber holder.

Aortic media. Aortas harvested from normal pigs were stored in Tris HCl 0.1 mol buffer, pH 7.4 with antibiotics (penicillin and streptomycin), and used within 4 days of harvesting as previously described (15–17). Before the experiment, the aorta was prepared by removing the loose adventitia. The intima and subjacent media were then removed by peeling off from one corner of the longitudinally opened aorta and discarded. This injury to the vessel wall provided a thrombogenic media adjacent to the adventitia that was exposed to circulating blood as 15 × 35-mm sections in the superfusion flow chamber experiments. For each experiment only one pig's aorta was used.

Quantitation of platelet deposition. The extent of platelet deposition on the aortic media segments was quantitated by assessing the amount of ^{111}In-labeled platelets interacting with the media. This was obtained by measuring the radioactivity in counts per minute (cpm) in each segment of aortic media after correction for radionuclide decay. The radioactivity per ml of blood was also determined at the time of the experiment. With knowledge of the whole blood platelet count (Coulter counter) obtained from the femoral artery, the number of platelets deposited on the media was calculated with the following equation:

$$\text{No. of deposited platelets} = \frac{(^{111}\text{In cpm in aortic media}) \times (\text{No. of platelets/ml blood})}{(^{111}\text{In cpm/ml blood})}.$$

Medication. Nitroglycerin solution (Montreal Heart Institute Formulary) was prepared by crushing and dissolving nitroglycerin tablets (Eli Lilly) in 5% dextrose in water, and the filtered solution (0.5 mg/ml) was infused into an ear vein with an intravenous pump (IVAC model 560). The therapeutic dose of nitroglycerin in pigs is unknown. Because an arbitrary dose may not be adequate or optimal in all pigs, and because there may be no correlation between the rate of nitroglycerin infusion and plasma nitroglycerin concentration, nitroglycerin was individually titrated to produce a 10% decrease in mean arterial pressure. Titration to this hemodynamic end point has also been used to indicate adequacy of treatment in several clinical studies (18) and has been shown to be associated with beneficial effects in humans, as well as with potent antithrombotic effects in vivo in pigs (9). In this study, the mean dose of nitroglycerin infused was 760 μg/min. The chamber runs were performed in duplicate 15 to 30 min after the nitroglycerin infusion was started.

Plasma cyclic GMP levels were measured with commercially available radioimmunoassay kits (Amersham International) after extraction by ethanol. Methylene blue (Sigma) was prepared at a concentration of 5×10^{-5} mol/liter and was given as a 10-ml bolus followed by an intravenous infusion of 3 ml/min with use of a Harvard pump. Serum

methylene blue level at the time of the superfusion experiment was determined spectrophotometrically. In two series of experiments, nitroglycerin was administered before infusion of methylene blue; in a third series, it was administered after the infusion.

The cyclic GMP analogue, 8-bromo cyclic GMP (Sigma), or its vehicle was infused with use of a Harvard pump through a side port in the arterial line just before it entered the superfusion chamber. The rate of the infusion was adjusted to yield a concentration of 7.4×10^{-6} mol/liter in the flow chamber.

Statistics. All values are expressed as mean value ± SE unless otherwise indicated. Analysis of variances was used to perform multiple group comparisons, and intergroup analysis was tested by the Fisher protected least significant difference test; p values of < 0.05 were considered significant.

Results

In these studies, each pig served as its own control. Thus, a baseline control platelet deposition level obtained before drug infusion was compared with the subsequent platelet deposition obtained after each drug infusion.

Nitroglycerin infusion. Despite a 10% decrease in mean arterial pressure in the pigs, blood flow through the ex vivo superfusion chamber was maintained constant with the peristaltic pump. This procedure was well tolerated by the animals. Under these constant flow conditions, platelet deposition was $55.5 \pm 6.1 \times 10^6$ on the aortic media, and intravenous nitroglycerin produced a decrease in platelet deposition that was 63.9 ± 10.4% ($n = 8$, $p = 0.01$) of the baseline control value. In four pigs in which plasma cyclic GMP levels were measured, there was a significant increase in cyclic GMP to a mean of 2,109.5 fmol/ml ($p < 0.05$) after nitroglycerin infusion, relative to a baseline level of 1,768.5 fmol/ml.

Methylene blue infusion before nitroglycerin administration. In a second group of eight pigs, infusion of the guanylate cyclase inhibitor, methylene blue, achieving a concentration of $2.1 \pm 0.4 \times 10^{-5}$ mol/liter, produced a nonsignificant trend toward an increase in platelet deposition to 155 ± 28% ($p = 0.09$) of the baseline control value. The subsequent administration of nitroglycerin intravenously caused no significant decrease in platelet deposition, in contrast to the previous experiments, and platelet deposition was 142 ± 35% of the baseline control value (p = NS) (Fig. 1).

Nitroglycerin, methylene blue and cyclic GMP administration. Relative to baseline control, the intravenous infusion of nitroglycerin before methylene blue administration in the third group of five pigs again resulted in a decrease in platelet deposition (Fig. 2), and confirmed the results documented initially. The subsequent infusion of methylene blue attenuated this effect of nitroglycerin with a mild increase in platelet deposition, which was now not significantly different from the baseline control value (Fig. 2). Infusion of the cyclic GMP analogue, 8-bromo cyclic GMP, thereafter resulted in a further significant decrease in platelet deposition to 16.4 ± 3.8% of control value ($p = 0.006$ vs. baseline control, and $p = 0.02$ vs. methylene blue infusion) (Fig. 2).

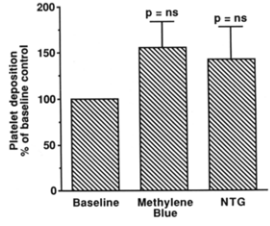
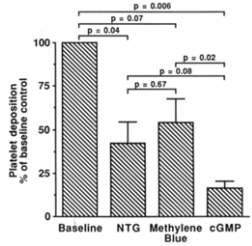


Figure 1. Platelet deposition expressed as a percent of the baseline control level is increased after methylene blue infusion and remained increased despite subsequent nitroglycerin (NTG) infusion. p values compare drug versus baseline.

Discussion

These results show that nitroglycerin can decrease platelet thrombus deposition on the exposed media of the injured vessel wall, and thus confirm our previous demonstration of its potent antithrombotic property noted in vivo (9). Under conditions of constant blood flow, nitroglycerin can decrease by as much as 57% the amount of platelet deposition onto the exposed aortic media relative to the baseline value. This antithrombotic effect of nitroglycerin was associated with a significant increase in plasma cyclic GMP levels. Pretreatment with methylene blue, to inhibit the guanylate cyclase enzyme (19), blocked this inhibition of mural platelet-

Figure 2. Platelet deposition expressed as a percent of baseline control is decreased after nitroglycerin (NTG) infusion. This decrease is attenuated by methylene blue infusion but enhanced by subsequent cyclic guanosine monophosphate infusion (cGMP).



thrombus deposition. However, despite methylene blue inhibition of the guanylate cyclase enzyme, increasing cyclic GMP levels by infusing the 8-bromo cyclic GMP analogue (20) markedly decreased platelet deposition. These findings indicate that the infusion of nitroglycerin to produce a 10% decrease in mean arterial pressure exerts a significant platelet inhibitory effect. This beneficial effect of nitroglycerin on the platelet–vessel wall interaction to decrease mural thrombosis (9) appears to be related to guanylate cyclase stimulation and cyclic GMP formation.

The dose range of nitroglycerin used in patients is extremely wide. Dose titration to a predetermined physiologic end point, such as a 10% decrease in mean arterial pressure, is commonly used in clinical studies to evaluate efficacy (18). This end point is sufficient to inhibit whole blood platelet aggregation in humans (11) and exerts a potent antithrombotic effect in pigs both in vivo (9) and, as shown in this study, ex vivo. Thus, in addition to vasodilation, at doses used clinically, nitroglycerin exerts significant platelet inhibitory and antithrombotic properties.

These studies were performed in the model of deep arterial injury exposing the media, where the thrombogenic stimulus is great and a well anchored mural platelet thrombus develops (21). This model is analogous to the rupture of an atherosclerotic plaque clinically (22) to produce a type 3 injury. The platelet inhibitory effect of nitroglycerin at clinically relevant doses has also been demonstrated in the presence of milder vessel injury associated with externally constricted coronary arteries and periodic formation and dislodgment of more labile platelet thrombi (14). However, in the presence of mild vessel injury not exposing the media and without external constrictors, nitroglycerin did not affect the adhesion of a monolayer of platelets to the vessel wall (9). Thus, nitroglycerin affects not only platelet interaction in vitro at high concentrations (6–8), but also platelet–vessel wall interaction in vivo at physiologic concentrations.

Mechanism of action. Earlier studies (6,8) using supraphysiologic concentrations of nitroglycerin in vitro demonstrated a platelet inhibitory effect that appeared to be related to a prostaglandin mechanism, either by decreasing platelet thromboxane A_2 or increasing prostacyclin production, or both. However, lower nitrate concentrations in vitro had no effect on prostacyclin production (12). Also, the beneficial clinical effects of nitroglycerin in vivo do not appear to be mediated by the prostaglandin system (13). However, both this study and the work of Folts et al. (14) not only confirm the antithrombotic efficacy of nitroglycerin in vivo at clinically relevant concentrations, but also suggest that this effect may be related to cyclic GMP generation. More recently, pretreatment with a cyclooxygenase inhibitor such as aspirin has been shown to potentiate the antiplatelet effect of nitroglycerin (23), probably by inhibiting platelets at two different levels. These recent studies raise the possibility that nitroglycerin may have a dual mechanism of action on platelets. At high concentration and in the absence of blood vessels, nitroglycerin may inhibit in vitro platelet aggregation through a prostaglandin mechanism. However, at physiologic concentration and in the presence of blood vessels, nitroglycerin may inhibit the platelet–vessel wall interaction through a cyclic GMP mechanism.

Pretreatment with methylene blue blocked the platelet inhibitory effect of nitroglycerin but this effect was only attenuated when methylene blue was given after the nitroglycerin infusion. This difference may occur because cyclic GMP production stimulated by nitroglycerin may produce levels that remain high despite subsequent inhibition of the guanylate cyclase enzyme by methylene blue. However, guanylate cyclase inhibition by methylene blue effectively prevents the stimulation of guanylate cyclase by nitroglycerin, as observed in the first and third groups of animals. The slight increase in platelet deposition after methylene blue infusion in group 2 pigs, although not statistically significant, perhaps because of the small number of animals, raises the possibility that the basal secretion of endogenous nitroglycerin in the form of endothelium-derived relaxing factor (24), a cyclic GMP modulator, may influence the reactivity of the circulating platelets or the thrombogenicity of the vessel wall.

Clinical implications. The beneficial antithrombotic effect of nitroglycerin in the presence of a type 3 injury exposing the very thrombogenic media, as demonstrated in this study at clinically relevant concentrations, may have important consequences. Continuous infusions of intravenous nitroglycerin are commonly used to treat unstable angina and appear to provide clinical benefit at doses that may not induce hemodynamic changes or long after tolerance to the hemodynamic effects of nitroglycerin have developed. Thus, the beneficial effects of exogenously administered nitroglycerin in the acute ischemic coronary syndromes (18), where platelet-thrombus formation may play an important pathophysiologic role, may in part be related to this antithrombotic property.

Both endothelium-derived relaxing factor and nitroglycerin produce their effects by generating nitric oxide (25,26). Thus, an important mechanism whereby the endothelium maintains its nonthrombogenicity may involve the endogenous release of endothelium-derived relaxing factor by the endothelium, similar to what was observed with exogenous nitroglycerin in this study. Endothelium-derived relaxing factor may contribute to the thromboresistance of the endothelium by stimulating guanylate cyclase and the production of cyclic GMP (26).

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